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Serial No. 10/507,303 : Group Art Unit 1641
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BRUSH-LIKE STRUCTURED SURFACE OF
POLY (ETHYLENE OXIDE) HAVING
ELEVATED DENSITY

DECLARATION

Commissioner for Patents
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Sir:

I, Iwao FURUYA, declare and say:

that I am presently residing at 20-10, Akane-cho, Kashiwa-shi, Chiba-ken
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that I am thoroughly conversant in both the Japanese and English languages;

that the attached document represents a true English translation of the certified
copy of Japanese Patent Application No. 2002-65298 filed March 11, 2002 which I
prepared.

I further declare that all statements made herein of my own knowledge are true
and that all statements made on information and belief are believed to be true; and
further that these statements were made with the knowledge that willful false
statements and the like so made are punishable by fine or imprisonment, or both,
under Section 1001 of Title 18 of the United States Code, and that such willful false
statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 15th day of March, 2006.

Iwao FURUYA



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(TRANSLATION)

JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of the following application as filed with this Office.

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Applicant(s): CENTER FOR ADVANCED SCIENCE AND TECHNOLOGY
INCUBATION, LTD.

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[List of Submitted Articles]

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[Name of Article]	Abstract	1
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[Proofing] Required

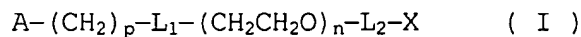
[Name of Document] Specification

[Title of the Invention] BRUSH-LIKE STRUCTURED SURFACE OF
POLY(ETHYLENE OXIDE) HAVING ELEVATED DENSITY

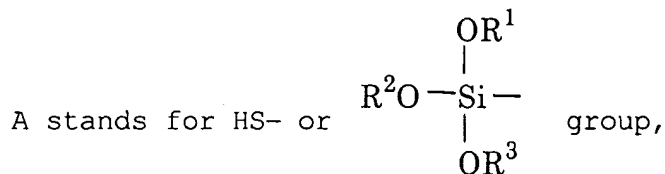
[Claims]

[Claim 1]

A surface which is characterized in that
(a) it is a biosensor surface to which at least one kind of the
polymers expressed by a general formula,



[in which



wherein R^1 , R^2 and R^3 each independently stands for a C_1-C_6
alkyl group,

L_1 stands for a first linker or a valence bond,

L_2 stands for a second linker or a valence bond,

X stands for a hydrogen atom, a functional group, a
protected functional group or a ligand,

p is an integer of 2 - 12, and

n is an integer of 10 - 1,000],

is linked via the A-moiety thereof, and that

(b), when each of said polymers is a polymer corresponding to the
polymer having HS- as A and $-CH_2CH_2CH(OCH_2CH_3)_2$ as $-L_2-X$ in said
general formula (I), said polymer is linked on a thin gold film

sensing chip of a surface plasmon resonance (SPR) detector, and the degree of adsorption of bovine serum albumin to said surface is measured with said SPR detector, the value of $\Delta\theta \times 10^{-4}$ [°] is 10 or less.

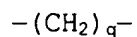
[Claim 2]

A surface according to Claim 1, in which the value of $\Delta\theta \times 10^{-4}$ [°] is 5 or less.

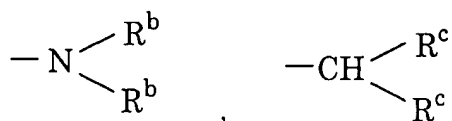
[Claim 3]

A surface according to Claim 1, in which, referring to the general formula (I): A is an HS group;

L_1 is $-\overset{\text{O}}{\parallel}{\text{C}}\text{O}-$ (which binds to ethylene oxide unit via the oxygen atom), $-\text{O}-$ or $-\text{S}-$; L_2 is a valence bond or



(wherein q is an integer of 2 - 6); and X is a hydrogen atom,



or $-\text{COOH}$, wherein R^b independently stands for a hydrogen atom or a $\text{C}_1\text{-C}_6$ alkyl, and R^c independently stands for a $\text{C}_1\text{-C}_6$ alkyloxy, or two R^c 's get together and form an oxy or an optionally $\text{C}_1\text{-C}_6$ alkyl-substituted ethylene group.

[Claim 4]

A surface according to Claim 1, in which, referring to said

general formula (I): A is a $(\text{CH}_3\text{O})_3\text{Si-}$ group; L_1 is $-\text{O}-$, $-\text{NHCOO}-$ (this group binds to an ethylene oxide unit via the oxygen atom) or $-\text{N}(\text{R}^d)-$ (wherein R^d stands for a $\text{C}_1\text{-C}_6$ alkyl); L_2 is a valence bond, $-(\text{CH}_2)\gamma-$ or $-\text{CO}(\text{CH}_2)\gamma-$ (wherein γ is an integer of 2 - 6); and X is a hydrogen atom,



or $-\text{COOH}$, wherein R^b independently stands for a hydrogen atom or a $\text{C}_1\text{-C}_6$ alkyloxy, and R^c independently stands for a $\text{C}_1\text{-C}_6$ alkyloxy, or two R^c 's get together and form an oxy or an optionally $\text{C}_1\text{-C}_6$ alkyl-substituted ethylene group.

[Claim 5]

A surface according to Claim 2, in which the support of said surface is made of a material selected from the group consisting of gold, silver, copper and aluminum.

[Claim 6]

A surface according to Claim 4, in which the support of said surface is made of a material selected from the group consisting of glass, a semiconductor, ceramics, metal oxide and alloy oxide.

[Claim 7]

A surface according to any one of Claims 1 to 6, in which at least two kinds of polymers expressed by said general formula (I) are linked in combination, one of which has an integer, n, of 10 -

60 and the other has an integer, n, of 50 - 1,000.

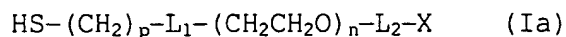
[Claim 8]

A surface according to any one of Claims 1 to 3, 5 and 7, in which said surface is formed on a sensing tip of said SPR detector.

[Claim 9]

A method for producing a surface according to Claim 1 to which a polymer having HS- as A in said general formula (I) is linked, said method comprising:

(A) a step of putting an aqueous solution of a polymer expressed by a general formula (Ia),



(wherein L₁, L₂, X, p and n have the same significations as defined as to said general formula (I) shown in Claim 1), in contact with a surface of a metal selected from the group consisting of gold, silver, copper and aluminum under the conditions sufficient to link a prescribed amount of said polymer to said metal surface, and thereafter washing away the unlinked polymer;

(B) a step of subsequently putting an aqueous solution of a polymer which may be identical to or different from said polymer linked to said metal surface in the above step and has a small value of the integer n (i.e., number of ethylene oxide units) in contact with said metal surface which has undergone the above step (A) under the conditions sufficient to link said polymer to said surface, and thereafter washing away the unlinked polymer; and

(C) a step of applying plural steps each of which is similar to the above step (B) to so obtained surface.

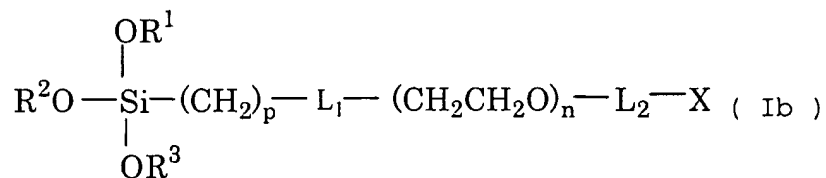
[Claim 10]

A method according to Claim 9, in which the step (B) and subsequent step or steps are carried out using a polymer differing from that used in the step (A); in the step (A) a polymer whose n in said general formula (I) is 50 - 1,000 being used and in the step (B) and subsequent step or steps a polymer whose n in said general formula (I) is 10 - 60 being used.

[Claim 11]

A method for producing a surface according to Claim 1 to which a polymer having a trialkoxysilyl group as A in said general formula (I) is linked, said method comprising:

(A) a step of putting an organic solvent solution of a polymer expressed by a general formula (Ib),



(wherein R^1 , R^2 , R^3 , L_1 , L_2 , X , p and n have the same significations as defined as to said general formula (I) shown in Claim 1),

in contact with a material selected from the group consisting of glass, a semiconductor, ceramics, metal oxide and alloy oxide under the conditions sufficient to adhere or link a prescribed amount of said polymer to the surface of said material, thereafter

distilling the solvent off, and washing away the unlinked polymer;

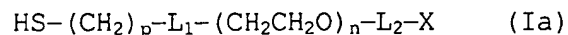
(B) a step of subsequently putting an organic solvent solution of a polymer which is identical to or different from said polymer linked to said material surface in the above step and has a small value of the integer n (i.e., number of ethylene oxide units) in contact with said material surface which has undergone the above step (A) under the conditions sufficient to adhere or link said polymer to said surface, thereafter distilling the solvent off, and washing away the unlinked polymer; and

(C) a step of applying plural steps each of which is similar to the above step (B) to so obtained surface.

[Claim 12]

A method for producing a surface according to Claim 1 to which a polymer having HS- as A in said general formula (I) is linked, said method including

(A') a step of putting an aqueous solution of at least two kinds of polymers, each of which is expressed by a general formula (Ia),



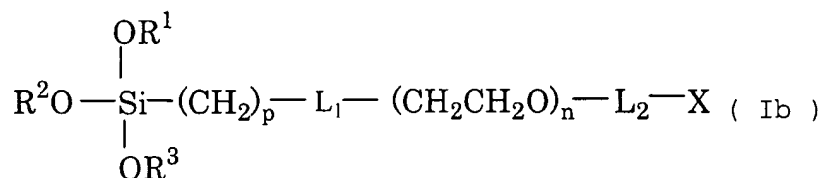
(wherein L_1 , L_2 , X, p and n have the same significations as defined as to said general formula (I) shown in Claim 1), and has an integer n which differs from each other by at least 10, in contact with a surface of a metal selected from the group consisting of gold, silver, copper and aluminum under the conditions sufficient to link prescribed amounts of said polymers to said metal surface, and thereafter washing away the unlinked

polymers.

[Claim 13]

A method for producing a surface according to Claim 1 to which a polymer having a trialkoxysilyl group as A in said general formula (I) is linked, said method including

(A') a step of putting an organic solvent solution of at least two kinds of polymers, each of which is expressed by a general formula (Ib),



(wherein R^1 , R^2 , R^3 , L_1 , L_2 , X , p and n have the same significations as defined as to said general formula (I) shown in Claim 1),

and has an integer n which differs from each other by at least 10, in contact with a material selected from the group consisting of glass, a semiconductor, ceramics, metal oxide and alloy oxide under the conditions sufficient to adhere or link prescribed amounts of said polymers to the surface of said material, distilling the solvent off, and washing away the unlinked polymers.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

This invention belongs to the art of biosensors. More

specifically, the invention relates to a biosensor surface which reduces or prevents non-specific adsorption or linkage thereto of impurities other than an intended analyte, which are contained in biological fluids or the like.

[0002]

[Prior Art]

As a means for detecting analytes present in biological samples, biosensors having a large variety of detection systems have been proposed. Of known biosensors, those utilizing surface plasmon resonance (SPR) are sensitive to changes in refractive index at surfaces and in the vicinities thereof of thin metal films (e.g., see A. Szabo, et al., Curr. Opin. Strnct. Biol. 5(1995) 699-705). The SPR enables an in situ observation of processes occurring between a surface and complex biological solutions and allows, e.g., acquisition of data from analytes in real time without requiring tagging of the analytes. It, therefore, is suitable for obtaining both kinetic and thermodynamic parameters, and hence an SPR sensor is one of those biosensors which are drawing attention.

[0003]

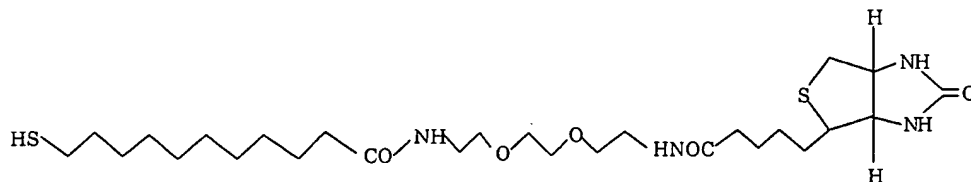
As a typical biosensor chip having this kind of surface, BIACORE[®] which is commercially available from Amersham Pharmacia Biotech., Inc. can be named, which is provided in the form of a chip in which a translucent matrix of dextran with carboxylated ends is immobilized on a thin gold film. A patent which is considered to claim such a detection surface is Japanese Patent No. 2815120 (corresponding to EP U. S. Patent No. 5,242,828 and EP 0 589 867B1) Gazette. This Official Gazette describes a surface formed by the steps of: linking organic molecules expressed by a

formula HS-R-Y (wherein R stands for a hydrocarbon chain which has a chain length exceeding ten atoms and may be interrupted with hetero atom(s), and Y stands for a ligand or an active group for covalently bonding a biocompatible porous matrix thereto) onto a membrane surface of the free metal such as gold, silver or the like via the thiol (or mercapto) groups therein; thereby covering the surface with a close-packed monolayer of the organic molecules; and thereafter covalently bonding to the surface a hydrogel as the biocompatible porous matrix, the hydrogel comprising agarose, dextran, polyethylene glycol and the like which may have functional group(s) for linking the ligand.

[0004]

Japanese Patent No. 3071823 (corres. to U. S. Patent No. 5,763,191 and EP 0 574 000B1) describes a surface formed by covalently bonding a hydrophilic linker (a straight chain molecule of 4 to 15 atoms in chain length) and a solid phase reactant (a biotin derivative residue) in the order onto a spacer molecule (a C₁ - C₃₀ alkylene chain) which links onto a support member via a sulfur atom (of a mercapto group). The same patent also describes a compound expressed by the following formula, as a typical biotinylated compound which forms such a surface:

[0005]



[0006]

The molecular chains on the surface which have a solid phase

reactant may further be diluted with diluent molecules which: do not have the solid phase reactant (i.e. in the above formula for example, the biotin derivative residue); or have neither the solid phase reactant nor a hydrophilic linker.

[0007]

Roberts et al., J. Am. Chem. Soc., 1998, 120, 6548-6555 describes a monolayer self-assembled on a gold surface via mercapto groups, using a compound based on an HS-spacer molecule (a C₁₁ alkylene chain)-hydrophilic linker (a chain formed of 3 or 6 ethylene oxide units). This document also teaches that a surface formed of a mixture of a compound whose hydrophilic linker portion consists of 3 ethylene oxide units and a compound whose hydrophilic linker portion consists of 6 ethylene oxide units (an oligopeptide ligand binding to its end) promotes cells' ligand-specific binding but reduces accumulation of proteins by so bound cells.

[0008]

Pavey et al., Biomaterials, 20 (1999) 885-890 discloses a surface formed by adhering various combinations of two kinds of poly(ethylene oxide)_n-poly(propylene oxide)_m-poly(ethylene oxide)_n triblock copolymers onto an SPR detecting thin metal film, with the suggestion that, on the surface thus formed, poly(ethylene oxide) chains would extend in the solution to form a brush-like architecture. Furthermore, the document shows that a surface onto which two kinds of the triblock copolymers having different poly(ethylene oxide) chain lengths (n) are adhered has generally lower protein (bovine serum albumin) adsorption than a surface onto which only one kind of the triblock copolymer is adhered.

[0009]

Meanwhile, among the foregoing prior art documents, Japanese Patent No. 2815120 discloses that a monolayer surface in which organic molecules are densely packed can be obtained by chemically adsorbing an organic compound whose chain (R) length exceeds 10 atoms, preferably is 12 - 30 atoms, e.g., 16-mercaptohexadecanol having a hydrophobic, considerably large alkylene chain, onto a metal surface via a thiol group. So obtained monolayer exhibits storage stability, and the patent furthermore suggests it may also play the role of a barrier layer effective in protecting the metal surface against chemical corrosion. Onto such a barrier layer, a hydrogel which minimizes protein compatibility and non-specific interaction is bound. Hence aforesaid BIACORE[®] (carrying hydroxygel of dextran) which likely is a preferred embodiment of the patented invention has been put into practical use. It is, however, by no means easy to have the barrier layer uniformly carry the hydroxygel and precise operations are required. Also, although non-specific adsorption of protein is considerably reduced, there is still room for further improvement.

[0010]

In Japanese Patent No. 3071823, when biotinyl (solid phase reactant) on the surface formed of aforesaid biotinylated compound is densely present, in order to improve slow binding of, for example, biotin and free avidin which forms non-covalently bonded pair with the biotin: either intentionally the biotinylated compound is sparsely bonded onto the surface of a support material; or both the diluent molecules and the molecules having a biotinyl residue (solid phase reactant) are bonded onto the surface by using the diluent molecules having no biotinyl residue

and the corresponding molecules having the biotinyl residue at a ratio of 10:1 - 2:1. Such a surface or that proposed by Roberts et al. has a hydrophilic linker portion formed of up to about 5 to 6 ethylene oxide units, but does not have such a hydrogel layer as described in Japanese Patent No. 2815120, and hence may cause non-specific adsorption of impurity proteins other than the object protein (e.g., streptavidin) or cells.

[0011]

Bavey et al. adheres the triblock copolymers onto a metal surface via their hydrophobic blocks, i.e., a poly(propylene oxide) domain, and it is difficult to obtain a surface with stability, uniformity and reproducibility like ordinary polymer coating, (cf. U. S. Patent No. 4,415,666). Moreover, it is also difficult to raise the density of poly(ethylene oxide) chains.

[0012]

Some of the present inventors, with the intent of providing a surface which eliminated or mitigated aforesaid shortcomings accompanying the prior art: dissolved a polymer formed mainly of poly(ethylene oxide) (which may hereafter be abbreviated as PEG) having a mercapto (-SH) group at one end and the other end being optionally protected in a buffer solution; and thereafter put the solution in contact with the gold surface of an SPR sensor chip for about an hour. As a result, they found that non-specific protein adsorption onto the surface could be reduced at least to the level equivalent to that onto the commercially available BIACORE[®] sensor chip surface CM5, and have filed a patent application directed to such a surface (cf. WO 01/86301).

[0013]

According to aforesaid Japanese Patent No. 2815120, R in an

HS-R-Y chain must be a hydrocarbon group having at least 10 atoms in order to closely pack the metal surface with the chains. Meanwhile, according to Japanese Patent No. 3071823, a biotinylated compound having a hydrophilic linker of a chain length of, for example, 4 - 15 atoms (1 - 3 of ethylene oxide units) is used and thereby a surface on which chains of the compound are sparsely linked is formed. Surprisingly, however, according to aforesaid WO 01/86301, macromolecules, which are entirely different from those polymers described in Japanese Patent No. 3071823 or Roberts et al. in that the macromolecules include long chain polymers each PEG domain of which has a molecular weight of 1,000 - 10,000, can be effectively linked to the metal surface via their mercapto groups in an aqueous solution. That is, in the case of such a polymer as described in WO 01/86301, a desired amount (or an adequate density) of the PEO chains is found to be stably linked to the surface in spite that it is recognized by those skilled in the art that a PEO layer which is hydrated so as to lower the interaction between the hydrophilic PEO chains and proteins at the surface through the spatial repulsion between the hydrophilic PEO chains and proteins in the solution is flexible and mobile.

[0014]

[Problems to be solved by the Invention]

If possible however, it will be desirable for such a surface as described in WO 01/86301 to be able to further reduce the non-specific adsorption of impurity proteins like a BIAcore® sensor surface. In this light, the present invention aims to provide a surface which can further reduce non-specific adsorption than the surface described in WO 01/86301.

[0015]

[Means of solving the Problems]

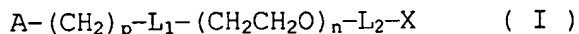
Although it is not intended to theoretically bind, probably because PEO chains in a solution are mobile and sterically repulse each other as stated above, it has been impossible by the linking method described in WO 01/86301 to significantly increase the linked amount of the polymer even if the processing time is extended. We have found, however, when the linking operations are repeated anew, additional polymer can be newly linked to the same surface. Furthermore, it has been confirmed that so obtained surface can significantly reduce the adsorption of impurity protein [e.g., bovine serum albumin (hereunder referred to as "BSA")] in comparison with the surface described in WO 01/86301.

[0016]

The present invention, therefore, provides a surface which is characterized in that

(a) it is a biosensor surface to which at least one kind of the polymers expressed by a general formula,

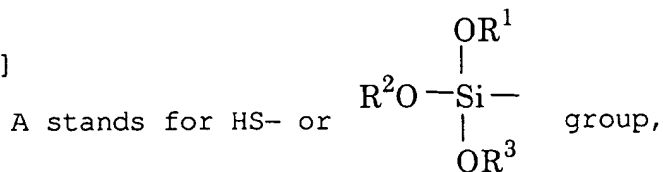
[0017]



[0018]

[in which

[0019]



[0020]

wherein R^1 , R^2 and R^3 each independently stands for a C_1 - C_6 alkyl group,

L_1 stands for a first linker or a valence bond,

L_2 stands for a second linker or a valence bond,

X stands for a hydrogen atom, a functional group, a protected functional group or a ligand,

p is an integer of 2 - 12, and

n is an integer of at least 10, preferably 20 - 1,000],

is linked via the A-moiety thereof, and that

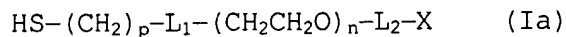
(b), when each of the polymers is a polymer corresponding to the polymer having HS- as A and $-\text{CH}_2\text{CH}_2\text{CH}(\text{OCH}_2\text{CH}_3)_2$ as $-\text{L}_2-\text{X}$ in the general formula (I), the polymer is linked on a thin gold film sensing chip of a surface plasmon resonance (SPR) detector, and the degree of adsorption of bovine serum albumin to the surface is measured with the SPR detector, the value of $\Delta\theta \times 10^{-4}$ [°] is 10 or less, preferably 5 or less.

[0021]

The present invention further provides a method for producing an embodiment of above-described surface, the method comprising:

(A) a step of putting an aqueous solution of a polymer expressed by a general formula (Ia),

[0022]



[0023]

(wherein L_1 , L_2 , X, p and n have the same significations as defined as to the foregoing general formula (I)), in contact with a surface of a metal selected from the group consisting of gold, silver, copper and aluminum under the conditions sufficient to link a prescribed amount of the polymer to the metal surface, and thereafter washing away the unlinked

polymer;

(B) a step of subsequently putting an aqueous solution of a polymer which may be identical to or different from the polymer linked to the metal surface in the above step and has a small value of the integer n (i.e., number of ethylene oxide units) in contact with the metal surface which has undergone the above step (A) under the conditions sufficient to link the polymer to the surface, and thereafter washing away the unlinked polymer; and

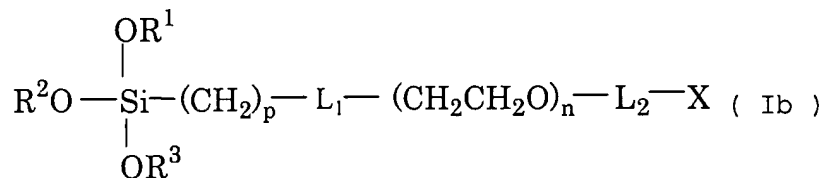
(C) a step of applying plural steps each of which is similar to the above step (B) to so obtained surface.

[0024]

The present invention furthermore provides a method for producing another embodiment of above-described surface wherein A moiety in the general formula (I) is a trialkoxysilyl group, the method comprising:

(A) a step of putting an organic solvent solution of a polymer expressed by a general formula (Ib),

[0025]



[0026]

(wherein R^1 , R^2 , R^3 , L_1 , L_2 , X , p and n have the same significations as defined as to the foregoing general formula (I)),

in contact with a material selected from the group consisting of glass, a semiconductor, ceramics, metal oxide and alloy oxide under the conditions sufficient to adhere or link a prescribed amount of the polymer to the surface of the material, thereafter

distilling the solvent off, and washing away the unlinked polymer;

(B) a step of subsequently putting an organic solvent solution of a polymer which is identical to or different from the polymer linked to the material surface in the above step and has a small value of the integer n (i.e., number of ethylene oxide units) in contact with the material surface which has undergone the above step (A) under the conditions sufficient to adhere or link the polymer to the surface, thereafter distilling the solvent off, and washing away the unlinked polymer; and

(C) a step of applying plural steps each of which is similar to the above step (B) to so obtained surface.

[0027]

As still another embodiment, the present invention provides a method for producing above-described surface, in which a mixture of at least two kinds of polymers each of which has an integer n differing from each other by at least 10, preferably 20, in the general formula (Ia) or (Ib) is used.

[0028]

[Embodiment for Carrying out the Invention]

The parameter, $\Delta\theta[^\circ]$, which indicates the degree of BSA adsorption onto a surface according to the present invention, is a value induced by change in refractive index at a metal interface, as described in, e.g., Jung et al., Langmuir, 1998, 14, 5636, and a larger value thereof indicates a greater amount of BSA adsorption. Here, this value varies in accordance with measuring conditions and a $\Delta\theta[^\circ]$ value described in this invention is based on the case where the value is measured under a "non-specific adsorption test" which will be described later.

[0029]

The term "when each of said polymers is a polymer corresponding to the polymer having HS- as A and $-H_2CH_2CH(OCH_2CH_3)_2$ as $-L_2-X$ in said general formula (I)" cited in the present specification signifies that the case where a polymer having other than HS- as A and other than $-CH_2CH_2CH(OCH_2CH_3)_3$ as $-L_2-X$ is substituted with a polymer having HS- and $-H_2CH_2CH(OCH_2CH_3)_2$ in the general formula (I) is assumed.

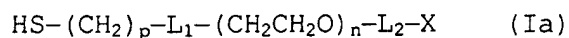
[0030]

While biosensor surfaces utilizing SPR are mainly conceived as the surfaces according to the present invention, the invention encompasses any other biosensor surfaces capable of tracing certain changes occurring at the surfaces due to so-called formation of a biologically specific non-covalent bond, namely the formation of a wide variety of coupled pair between a ligand and a receptor, e.g., an antigen or a hapten and an antibody, sugar and lectin, a substrate and an enzyme, a hormone and a receptor thereof, oligonucleotide and a complementary chain thereof, and the like. As the traceable signals, they may be changes in radioactivity, contact angle, sedimentation, UV spectrum, surface plasmon resonance or the like.

[0031]

Most of the polymers useful for the surfaces of the present invention are publicly known already. Then a polymer in which A represents HS- is described in aforementioned WO 01/86301 and the polymer is expressed by the general formula (Ia),

[0032]



[0033]

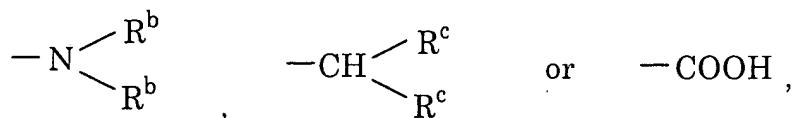
(wherein L_1 , L_2 , X, p and n have the significations as previously

defined). As L_1 , L_2 , and X , the optimum groups can be selected respectively in accordance with the production method. L_1 and L_2 may independently be a valence bond or various kinds of linkers. Typical examples of L_1 as a concrete linker are $-\text{COO}-$ (which group binds to an ethylene oxide unit via the oxygen atom), $-\text{O}-$ and $-\text{S}-$. An example of L_2 as a concrete linker is $-(\text{CH}_2)_q-$ (here q is an integer of 2 - 6).

[0034]

X stands for a hydrogen atom, a functional group or a protected functional group, which functional group may be any group which is capable of covalently bonding with the ligand. In the case where the ligand is a protein or nucleotide for example, the functional group or protected functional group may be

[0035]



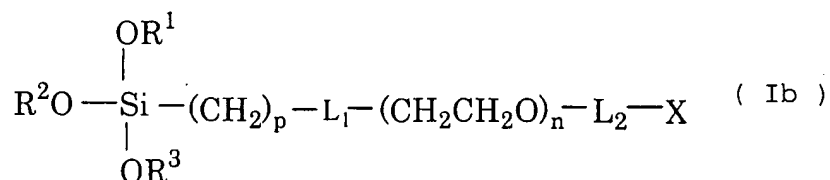
[0036]

wherein R^b independently stands for a hydrogen atom or a $\text{C}_1\text{-C}_6$ alkyl, and R^c independently stands for a $\text{C}_1\text{-C}_6$ alkyloxy (ketal), or two R^c 's may get together and stand for an oxy (in the case the group as a whole becomes an aldehyde group: $-\text{CHO}$), or R^c may be an optionally $\text{C}_1\text{-C}_6$ alkyl-substituted ethylene group (forming a cyclic ketal). In particular, the aldehyde group or the protected aldehyde group (ketal group) can be conveniently used. The $\text{C}_1\text{-C}_6$ alkyl specifically is methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, n-hexyl and the like, methyl being preferred.

[0037]

Again, most of the polymers expressed by the general formula (Ib) in which A is a trialkoxysilyl group,

[0038]



[0039]

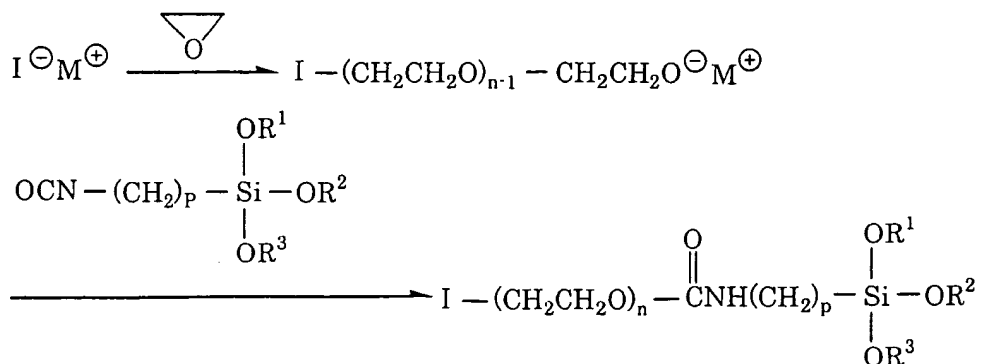
(wherein R^1 , R^2 , R^3 , L_1 , L_2 , X , p and n have the significations as previously defined),
are publicly known already.

[0040]

More specifically, a C_1 - C_6 alkyl in the definition of R^1 , R^2 , and R^3 has the same signification as that in the definition of R^b and R^c . Also in the general formula (Ib), $-\text{X}$ may have the same signification as X in the general formula (Ia); L_1 is, for example, $-\text{O}-$, $-\text{NHCOO}-$ (binding to an ethylene oxide unit via the oxygen atom) or $-\text{N}(\text{R}^d)-$ (R^d being a C_1 - C_6 alkyl); and L_2 is $-(\text{CH}_2)_\gamma-$ or $-\text{CO}(\text{CH}_2)_\gamma-$ (γ being an integer of 2 - 6). Such polymers can be conveniently produced according to, e.g., any of the following reaction schemes.

Reaction scheme 1:

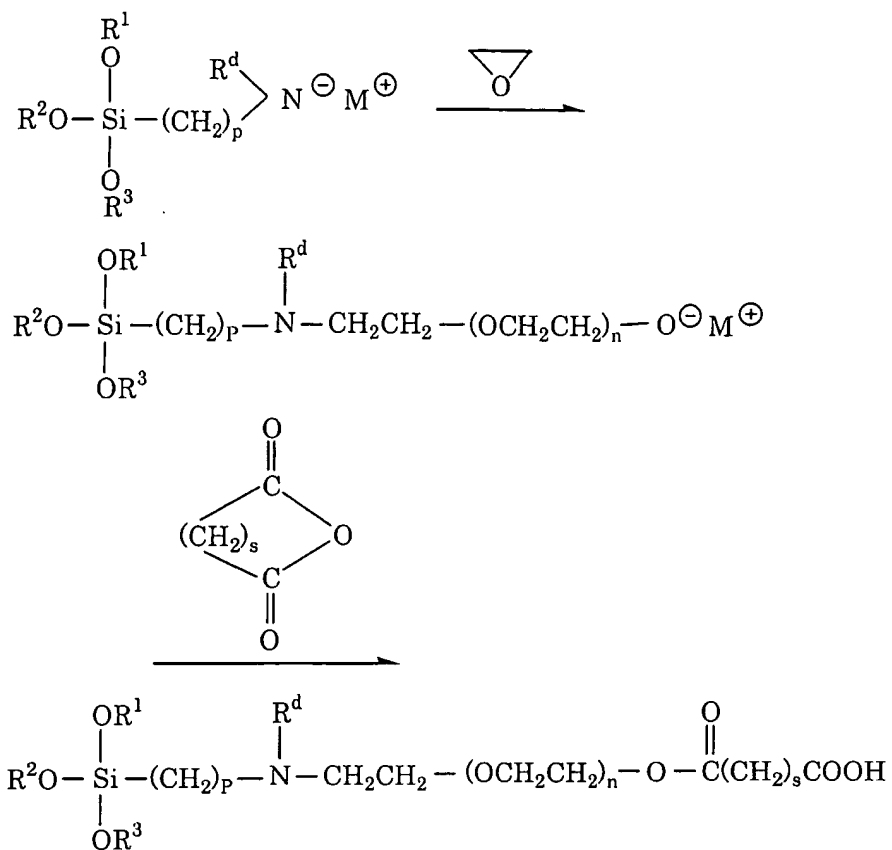
[0041]



[0042]

Reaction scheme 2:

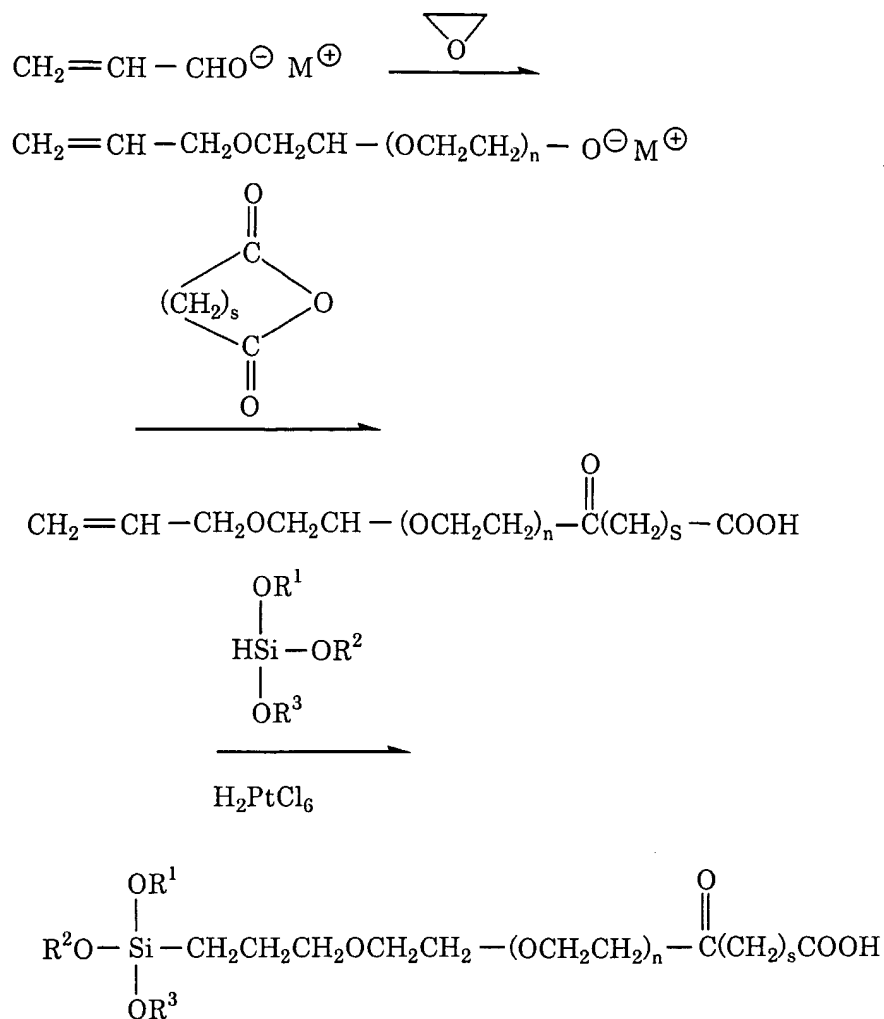
[0043]



[0044]

Reaction scheme 3:

[0045]



[0046]

(wherein M stands for potassium, sodium or lithium).

The foregoing living polymerization steps can be carried out under publicly known reaction conditions (e.g., see WO96/32434, WO97/06202, etc.). Otherwise, they can be carried out following the working Examples given later, or by modifying the conditions described therein.

[0047]

Although not in any limitative sense, when a surface is

formed using aforementioned polymer expressed by the general formula (Ia), the support of the surface (a thin metal film in the case of an SPR sensor) is preferably selected from such metals as gold, silver, copper, aluminum and the like. Meanwhile, when the polymer expressed by the general formula (Ib) is used, the support is preferably selected from glass, a semiconductor, ceramics, metal oxide and alloy oxide, which form hydroxyl groups on the surface with moisture or the like and form covalent bonds with trialkoxysilane.

[0048]

A surface according to the present invention is in the state where the amount of linked polymer is significantly increased in comparison with the amount of the linked polymer resulted from single treatment by subjecting the polymer expressed by the general formula (Ia) or (Ib) either to polymer-linking treatments plural times using a solution containing the polymer or to a linking treatment using a solution containing at least two kinds of polymers having different sizes of ethylene oxide units in spite that a nearly constant saturated linkage group is obtained in the case of single linking treatment of substantially identical polymers. When a polymer expressed by the general formula (Ia) is used, the polymer is dissolved in a suitably buffered aqueous solution, and the solution is put in contact with the support surface at an adequate temperature, e.g., ambient temperature, (20-37°C). The optimum polymer concentration of the polymer solution varies in accordance with the molecular weight of the polymer used but is usually 0.1 - 5 mg/ml, preferably 1 mg/ml. Then the contact is carried out by putting the support surface in contact with the polymer solution and incubating them for several

tens of minutes to several hours.

[0049]

Thus a fixed amount of the polymer is linked (presumably by chemical bonding) to the support surface. Unlinked polymer is removed from so obtained surface by washing the surface. Any washing liquid can be used so long as it can effectively remove the unlinked polymer, while the use of diluted NaOH aqueous solution is preferred. After the completion of the washing, the surface is subjected to another polymer-linking treatment using a polymer solution anew. This second linking treatment may be substantially identical with the first linking treatment including the washing. Preferably, the second linking treatment and washing are repeated at least one more time. The polymer used in the second and subsequent linking treatments may be identical with that used in the first treatment, or may be in each time a polymer having a poly(ethylene oxide) block of a molecular weight different from the case of the first treatment. When their molecular weights are different from each other, preferably the polymer used in the second treatment is selected so as to have a smaller molecular weight than polymer used in the first treatment. The polymer may be selected so that the molecular weight is gradually reduced as the linking treatment is repeated. Although not in limitative sense, preferred combination of polymers used in the first linking treatment and the second and subsequent linking treatments, based on the molecular weight of the poly(ethylene oxy) block, comprises: a polymer expressed by the general formula (I) or (Ia) wherein the integer n is 50 - 1,000 in the first linking treatment; and another polymer expressed by the same wherein the integer n is 10 - 60, preferably 20 - 60 in the second

and subsequent linking treatments.

[0050]

When the polymer of the general formula (Ia) is replaced with a polymer of the general formula (Ib), the polymer is dissolved preferably in an anhydrous organic solvent (for example, an easily polymer-soluble organic solvent such as toluene) and the solution is used for a linking or adhering treatment of the polymer to a surface of, for example, glass, titanium, aluminum or the like, thereafter the solvent is distilled off, and the polymer which is unreactive with the surface is washed away with the same organic solvent to form the intended surface, all other features being the same as those in the case of using a polymer of the general formula (Ia).

[0051]

On a surface thus formed, a high polymer brush-like architecture of polyethylene glycol [or poly(ethylene oxide)] having a higher density can be formed in comparison with a surface obtained by single polymer-linking treatment and thereby it is possible to markedly reduce the adsorption of impurity proteins in a sample which induce non-specific adsorption onto a biosensor surface. Further, the surface will make it possible to detect specific interactions between biological molecules with a higher degree of accuracy.

[0052]

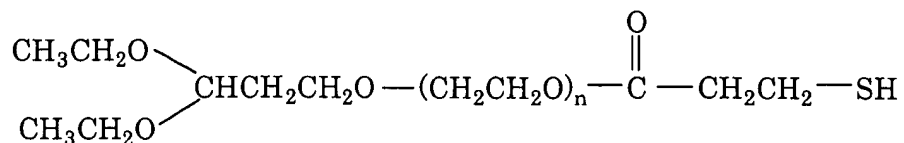
[Examples]

The present invention is hereinafter explained further in detail, referring to specific examples. However, those examples are not intended to limit the present invention in any way.

Polymer Production Example:

Synthesis of acetal-PEG-SH (Mn=2000, 5000)

[0053]



[0054]

Distilled tetrahydrofuran (THF) 20 ml and 3,3-diethoxy-1-propanol, an initiator, 0.2 mmol (0.032 ml) were contained in an argon-substituted reactor, and further an equivalent amount of potassium naphthalene was added thereto, followed by 15 minutes' stirring to conduct metallization. Then ethylene oxide 22.7 mmols (1,135 ml) was added, followed by two days' stirring at room temperature to conduct the polymerization. As a reaction-suspending agent, N-succinimidyl-3-(2-pyridylthio)propionate (SPDP) 0.4 mmol (0.125 g) was dissolved in a small amount of distilled THF and into the resultant solution aforesaid polymerization reaction solution was dropped under cooling with ice through an isopiestic dropping funnel. After an overnight's stirring, the reaction was suspended and the polymer was recovered through the processes of washing with saturated saline solution, extraction with chloroform, reprecipitation from ether and lyophilization with benzene. The construction of the recovered polymer was confirmed with ^1H -NMR, and the amount of SPDP residue introduced into the polymer terminals was also confirmed by UV absorption of 2-thiopyridone which was released upon reaction with 2-mercaptoethanol.

[0055]

PEG-SS-Py 2.0×10^{-2} mmol (100 mg) was dissolved in 4 ml of distilled water, to which dithiothreitol 0.1 mmol (15.42 mg), five times the above amount in mol, was further added, followed by 30 minutes' stirring at room temperature. After the reaction, a polymer whose PEG segment had $M_n=5000$ (hereafter abbreviated as PEG 5000) was recovered through the processes of washing with saturated saline solution, extraction with chloroform and reprecipitation from ether. The construction of the recovered polymer was confirmed with $^1\text{H-NMR}$ and further the amount of the terminal SH groups was determined by the reaction with 2-pyridyldisulfide (2-PDS).

[0056]

Further, substantially the same operations as above were repeated except that the feed amount of the ethylene oxide was decreased and a polymer with a PEG segment of $M_n=2000$ was produced. The polymer is hereafter abbreviated as PEG 2000. "Mn" herein means the molecular weight of the PEG segment.

Examples 1 - 8: Immobilization of PEG onto JI sensor chips

A solution containing each of the polymers obtained in the above production examples at a concentration of 1.0 mg/ml [solvent: 1M NaCl-containing 50 mM PBS (pH7.3)] was applied to the gold surface of a JI sensor chip (acquired from BIACORE) at a flow rate of 20 $\mu\text{l}/\text{min}$. for 20 minutes at 37°C. Thereafter the surface was washed twice with a 50 mM NaOH solution at a flow rate of 20 $\mu\text{l}/\text{min}$. for 30 seconds. A series of the operations was defined as one cycle of the immobilization experiment and various surfaces were obtained by immobilizing various polymers under the following conditions, respectively.

Example 1 (control):

PEG 5000 (1) ...one cycle immobilization of PEG 5000 on the gold surface, parenthesized numeral indicating the cycle number of the immobilization experiment

[0057]

Example 2 (control):

PEG 2000 (1) ...one cycle immobilization of PEG 2000 on the gold surface

Example 3 (present invention):

PEG 5000 (1) + PEG 2000 (1) ...one cycle immobilization of PEG 5000 on the gold surface, followed by one cycle immobilization of PEG 2000 on the same gold surface

Example 4 (present invention):

PEG 5000 (1) + PEG 2000 (3) ...one cycle immobilization of PEG 5000 on the gold surface, followed by three cycles of PEG 2000 immobilization on the same gold surface

Example 5 (present invention):

PEG 5000 (1) + PEG 2000 (5) ...one cycle immobilization of PEG 5000 on the gold surface, followed by five cycles of PEG 2000 immobilization on the same gold surface

Example 6 (present invention):

PEG 5000 and PEG 2000 (1) ...one cycle immobilization of a PEG 5000 and EG 2000 mixture on the gold surface, using a solution of the mixture

Example 7 (present invention):

PEG 5000 (4) ...four cycles of PEG 5000 immobilization on the gold surface

Example 8 (present invention):

PEG 5000 (4) + PEG 2000 (3) ...four cycles of PEG 5000 immobilization on the gold surface, followed by three cycles of

PEG 2000 on the same gold surface

Example: Non-specific Adsorption Test

To each of the surfaces of the JI sensor chips obtained in Examples 1 - 8, an untreated CM5 (formed by adsorbing carboxymethyldextran (acquired from BAIACORE) onto a JI sensor chip) and a blocked CM5 (formed by blocking the carboxymethyl groups), bovine serum albumin (BSA) solution at a concentration of 1 mg/ml [solvent: 0.15 M NaCl-containing 10 mM HEPES buffer solution (pH 7.4) + 3 mM EDTA and 0.005% (v/v) surfactant P20] was applied at a flow rate of 20 μ l/min. for 10 minutes at 25°C.
[0058]

Three (3) minutes after the end of injection of the BSA solution, the amount of BSA linked to each of the surfaces was measured. Here, the untreated CM5 was used as it was purchased from the market. Meanwhile, the blocked CM5 was prepared through the steps of ① applying a NHS/EDC mixed solution to the surface of the untreated CM5 at a flow rate of 10 μ l/min. for 10 minutes, and successively ② applying an ethanolamine solution at a flow rate of 10 μ l/min. for 10 minutes, and finally applying a 50 mM NaOH solution at a flow rate of 10 μ l/min. for 1 minute as a washing process. The last washing was conducted three times to block the carboxymethyl groups. The results are shown in Fig. 1.
[0059]

From Fig. 1, it can be understood that BSA adsorption onto a surface according to the present invention is markedly lower than the commercially available untreated CM5 and blocked CM5, and moreover than Example 1 (control) and Example 2 (control).

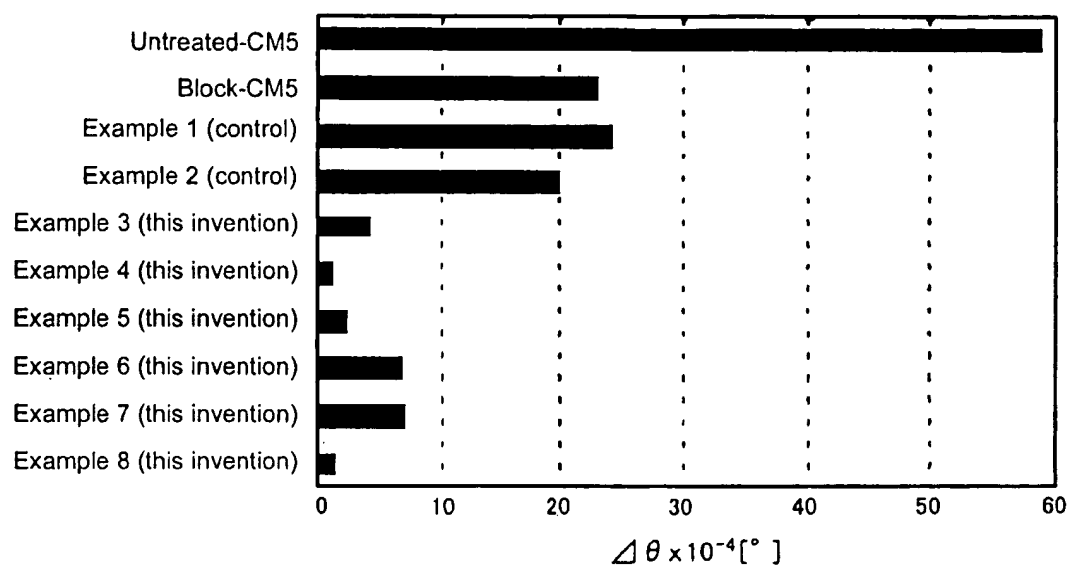
[Brief Description of the Drawings]

[Figure 1]

Fig. 1 is a graph showing the results of the BSA adsorption tests given to surfaces.

[Name of Document] Drawing

[Figure 1]



[Name of Document] Abstract

[Abstract]

[Problem to be solved by the invention]

To provide a biosensor surface which reduces non-specific adsorption thereto.

[Means of solving the problem]

A surface on which the adsorption of protein (e.g., BSA) is markedly reduced, the surface being produced by putting a solution of polymers mainly composed of poly(ethylene oxide) having binding sites to a sensor surface in contact with the biosensor surface plural times to link the polymers.

[Selected Drawing] None

[Name of Document] Notice of Change of Applicant's Name

[Date of Filing] June 19, 2002

[Addressed to] Commissioner, Japan Patent Office

[Indication of the Case]

[Application Number] Patent Application No. 2002-65298

[Successor]

[Identification Number] 899000024

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[Name] Heikichi ODAJIMA

[Indication of Official Fee]

[Deposit Number for Prepayment] 019666

[Amount of Payment] 4,200 Yen

[Others]

A deed of assignment and power of attorney were filed in the
form of a supplementary document of the same date.

[Proofing] Required

[Name of Document] Supplementary Document

[Date of Filing] June 19, 2002

[Addressed to] Kozo OIKAWA, Commissioner, Japan Patent Office

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[Application Number] Patent Application No. 2002-065298

[Person making Supplement]

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[Name of Document to be Supplemented] Notice of Change of

Applicant's Name

[Content of Supplement] Deed of Assignment and Power of Attorney

[List of Submitted Articles]

[Name of Article] Deed of Assignment 1

[Name of Article] Power of Attorney 1

DEED OF ASSIGNMENT

March 11, 2002

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This is to certify that I hereby assigned to you, the right
to obtain a patent on the invention mentioned below.

1. Patent application number: Patent Application No. 2002-065298
2. Title of the invention: BRUSH-LIKE STRUCTURED SURFACE OF
POLY(ETHYLENE OXIDE) HAVING ELEVATED DENSITY

POWER OF ATTORNEY

March 11, 2002

We, undersigned, hereby appoint :

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